U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER
ABERDEEN PROVING GROUND, MD 21010-5424

CCDC CBC-TR-1571

DARPA Antibody Technology Program, Phase II: Characterization of an Anti-HA33A Human Immunoglobulin G Antibody Produced by AnaptysBio, Inc.

Patricia E. Buckley
Alena M. Calm
Heather Welsh
Roy Thompson
Darrel Menking
James Carney

RESEARCH AND TECHNOLOGY DIRECTORATE

Candice Warner
Melody Zacharko

EXCET, INC.
Springfield, VA 22150-2519

June 2019

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-06-2019	Final	Sep 2012–Sep 2015

4. TITLE AND SUBTITLE
DARPA Antibody Technology Program, Phase II: Characterization of an Anti-HA33A Human Immunoglobulin G Antibody Produced by AnaptysBio, Inc.

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Buckley, Patricia E.; Calm, Alena M.; Welsh, Heather; Thompson, Roy; Menking, Darrel; Carney, James (CCDC CBC); Warner, Candice; and Zacharko, Melody (Excet)

5d. PROJECT NUMBER
BA08DET000/1R3Z11

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, CCDC CBC, ATTN: FCDD-CBR-BC, APG, MD 21010-5424
Excet, Inc., 6225 Brandon Avenue, Suite 360, Springfield, VA 22150-2519

8. PERFORMING ORGANIZATION REPORT NUMBER
CCDC CBC-TR-1571

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Advanced Research Projects Agency, 675 North Randolph Street, Arlington, VA 22203-2114

10. SPONSOR/MONITOR'S ACRONYM(S)
DARPA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES
The U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC) was formerly known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

14. ABSTRACT:
The Defense Advanced Research Projects Agency (DARPA; Arlington, VA) Antibody Technology Program (ATP) focused on development of technologies that enhance the thermal stability and binding affinity of a given antibody. The U.S. Army Edgewood Chemical Biological Center (ECBC; now known as U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) functioned as an independent laboratory to provide technical support on immune reagents and assist in defining the government-supplied antibody–antigen pairs. Project goals were to (1) implement standardized methods for characterizing antibodies developed at ECBC with de novo thermal and binding properties of select reagents for use by DARPA-funded investigators, and (2) use those methods to validate changes in antibody thermal stability and binding affinities achieved by DARPA investigators. Because combinatorial approaches to antibody enhancement are random and may lead to fortuitous improvements in stability or affinity, ATP enhancement strategies were required to be transferable to other antibody molecules. The Bot 56 antibody, which detects HA33 of BOTA, was chosen for enhancement. The focus was evaluation of the Bot antibody supplied by AnaptysBio, Inc. (San Diego, CA) for affinity and stability enhancements. Results of this study include standardized parametric data on antibody properties and performance.

15. SUBJECT TERMS

Antibody Technology Program (ATP)	HA33A antibody	Botulinum neurotoxin
Defense Advanced Research Projects Agency (DARPA)	Biosurveillance	Quality testing
Enzyme-linked immunosorbent assay (ELISA)	Hemagglutinin 33/A (HA33A)	

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Renu B. Rastogi
U	U	U	UU	32	**19b. TELEPHONE NUMBER** *(include area code)* (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was supported by the Defense Advanced Research Projects Agency (Arlington, VA), project number BA08DET000/1R3Z11. The work was started in September 2012 and completed in September 2015.

At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center was known as the U.S. Army Edgewood Chemical Biological Center.

This report has been approved for public release.

Blank

CONTENTS

FIGURES

TABLES

DARPA ANTIBODY TECHNOLOGY PROGRAM, PHASE II: CHARACTERIZATION OF AN ANTI-HA33A HUMAN IMMUNOGLOBULIN G ANTIBODY PRODUCED BY ANAPTYSBIO, INC.

1. INTRODUCTION

In an effort to more fully characterize and compare the physical and functional properties of antibody reagents in its repository, the Joint Product Management Office for Biosurveillance (Fort Detrick, MD) has instituted a quality program for the standardization of test methods. Production methods for antibodies used in detection devices have drastically changed over time. Animal models provide the most common method for producing polyclonal antibodies; however, the antibodies lack antigen-binding specificity, and the antibody affinity depends on the individual animals. Development of the monoclonal antibody (mAb) allowed for more specificity; however, the use of large numbers of animals is still required. With the advent of hybridoma cell culture production, larger quantities of high-activity antibodies were produced, and existing cell lines could be panned for higher-affinity antibodies. Recombinant methods have greatly increased researchers' ability to produce more-specific antibodies with antigen-binding fragment (Fab), single-chain variable fragment (scFv), and single-domain antibody (sdAb). Many varying recombinant production systems are being used, from basic bacteria, yeasts, and filamentous fungi to insect cell lines and mammalian cells, and including transgenic plants and animals (*1*).

The Defense Advanced Research Projects Agency (DARPA; Arlington, VA) Antibody Technology Program (ATP) focused on the development of technologies that enhance the thermal stability and binding affinity of a given antibody. Increased thermal stability would eliminate the need for cold storage as well as increase the usability of antibodies in harsh conditions, such as those experienced by troops in the field. Increasing binding affinity also allows for the development of multiplex sensors that can detect a greater variety of antigens. The U.S. Army Edgewood Chemical Biological Center (ECBC; now known as the U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) functioned as an independent testing laboratory for this program. ECBC personnel provided specific technical support on immune reagents as well as assistance in defining the government-supplied antibody–antigen pairs. Standardized methods developed at ECBC for characterizing antibodies were used for validating the changes in antibody thermal stability and binding affinity that had been achieved by DARPA investigators. Because combinatorial approaches to antibody enhancement are random and may lead to fortuitous improvements in stability or affinity, the strategies for ATP enhancement were required to be directed so that they could be transferable to other antibody molecules.

The primary objectives for the Phase I ATP were to develop and demonstrate strategies that independently modulate antibody stability and affinity in an antibody molecule that was provided by the U.S. Government. The Phase II goal was to modify an antibody using the Phase I techniques and thereby produce 2 g of a single protein that yielded a 100-fold increase in the affinity and a 10 °C increase in the melting temperature of a provided protein.

The focus of the work highlighted in this report is the evaluation of the anti-Bot antibody, supplied by the DARPA-funded investigator AnaptysBio, Inc. (San Diego, CA) for affinity and stability enhancements.

2. MATERIALS AND METHODS

2.1 Antibody and Antigen

2.1.1 Choice of Antibody

The Critical Reagents Program collection contained a large number of both polyclonal and monoclonal anti-botulinum toxin antibodies. For this project, the recombinant Bot 56 antibody, which detects hemagglutinin 33/A (HA33A), a part of the botulinum neurotoxin complex type A, was chosen.

2.1.2 Choice of Antigen

The antigen, hemagglutinating protein HA33A from *Clostridium botulinum*, is resistant to protease digestion and likely protects the neurotoxin from activation. Recombinant HA33A plasmids were transformed via electroporation into BL21(DE3) *Escherichia coli* cells (Novagen; EMD Biosciences; Madison, WI). A single colony was used to inoculate 1 mL of Luria broth (LB; Thermo Fisher Scientific; Madison, WI) containing 100 µg/mL kanamycin (kan; Thermo Fisher Scientific), which was incubated overnight (37 °C, 300 rpm). In the morning, the culture was scaled up to 50 mL of LB and incubated (37 °C, 225 rpm) until it reached log phase. The log-phase culture was scaled up by adding 10 mL to 500 mL of terrific broth media (Novagen) that contained 100 µg/mL kan. The cultures were grown for 20 h, the cell paste was collected via centrifugation, and the paste was mechanically lysed with an M110P microfluidizer (Microfluidics Corporation; Newton, MA) at 20,000 psi. HA33A was purified over a nickel affinity column on an ÄKTAxpress system (GE Healthcare Life Sciences; Marlborough, MA) and desalted into 50 mM citric acid (pH 4.0). The purified protein was minimally biotinylated using an EZ-Link sulfo-NHS-biotinylation kit (Thermo Fisher Scientific) for use with horseradish peroxidase (HRP)-labeled streptavidin in the enzyme-linked immunosorbent assay (ELISA).

2.1.3 Modified Anti-HA33A Human Immunoglobulin G (IgG) Production

The Bot 56 antibody was modified by AnaptysBio as previously described (*2*). Complementarity-determining regions of defined specificity were grafted onto a known stable framework, human IgG. This modified anti-HA33A human IgG antibody is designated in this report as APE 533.03.

2.2 Test Methods

For this work, we used standardized parametric tests that were established during the MS2 scFv antibody DARPA ATP Phase I study (*3*).

2.2.1 Antibody Concentration Measurement

The concentrations of APE 533.03 and Bot 56 were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). This instrument measured the absorbance of light at 280 nm (A_{280}) for each sample. Extinction coefficients were used in conjunction with A_{280} values to determine accurate concentrations. The concentration of each sample was determined by dividing the average A_{280} value by the extinction coefficient for IgG (1.4) or Bot 56 Fab (1.77). Each reading required a 2 µL sample, which was placed on the sample pedestal. The instrument was blanked using phosphate-buffered saline (PBS; Sigma-Aldrich; St. Louis, MO), and readings were taken in triplicate. As a positive control and to validate instrument operation, bovine γ-globulin (BGG; Bio-Rad; Hercules, CA) was also tested.

2.2.2 Molecular Weight and Purity Measurements

An Experion automated electrophoresis system (Bio-Rad) was used to determine the molecular weight and purity of APE 533.03 and Bot 56. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software, is designed to accomplish separation, staining, destaining, detection, and basic data analysis. Sample purity was determined by calculating the percent mass of the separated proteins in a sample. For Experion analysis, each antibody concentration was standardized by dilution in PBS to a final concentration of 1 mg/mL. The control (BGG) and the antibody samples were then processed using the validated procedure specified in the *Experion Pro260 Analysis Kit Instruction Manual* (*4*). Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. The sample was reduced with dithiothreitol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip. The instrument was operated via the Experion software. All samples were run in triplicate alongside one sample of the control (BGG) and the Pro260 ladder. Analysis was performed using the Experion software.

2.2.3 Protein Behavior Measurement

Dynamic light scattering (DLS) was used to evaluate how the proteins behaved in solution. Three tests were performed to determine protein uniformity in solution. Polydispersity is a measure of the size distribution of particles in solution. Protein molecules that have a polydispersion value of less than 20% are considered monodisperse. The hydrodynamic radius and molecular weight of the sample are displayed by two graphs. The correlation graph indicates the relative particle size, and the steepness of the line indicates the monodispersion level of the sample. The regularization graph (derived from data) shows the hydrodynamic radius, percent mass, and molecular weight. The DLS software uses prediction algorithms to produce this range of values for the protein under evaluation.

For DLS analysis, five 20 µL aliquots of the antibody along with the control (bovine serum albumin; Sigma-Aldrich) were placed into a quartz 384-well plate (Wyatt Technology Corp.; Santa Barbara, CA) and centrifuged (2 min, 239×*g*) to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation, and the plate was then placed into a temperature-controlled

DynaPro plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Results were averaged, and the Wyatt Technology Dynamics software was used to measure polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample. The results for three wells were averaged and reported.

2.2.4 Thermal Stability Measurement

Differential scanning calorimetry (DSC) was used to obtain a quantitative melting temperature (T_m) for each of the antibody proteins. The T_m was determined to predict results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C predicts that antibody activity after the thermal stress test will remain above 50%. A T_m below 70 °C predicts, at minimum, a 50% decrease in antibody activity after the thermal stress test. For DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS at pH 7.4. Samples were degassed for 5 min before analysis and injected into the sample cell of a VP-DSC calorimeter (MicroCal; Northampton, MA). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all experiments. The samples were scanned in duplicate from 15 to 100 °C at a rate of 60 °C/h. The transition midpoint T_m of the protein was determined by data analysis with Origin 7.0 software (MicroCal).

2.2.5 Thermal Stress Testing

All samples were diluted to a concentration of 1 mg/mL before heat was applied to negate protective effects due to concentration. Antibodies were diluted to 1 mg/mL in 1× PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and was marked time 0. The remaining four aliquots were heated to 75 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. These samples were then tested for activity using ELISA.

2.2.6 ELISA Methodology

After thermal testing was complete, ELISAs were performed in triplicate using the standard capture assay techniques. Each antibody sample was diluted to 20 µg/mL in PBS. A twofold serial dilution was performed across each Nunc MaxiSorp 96-well plate (Thermo Fisher Scientific). Samples were incubated at 4 °C overnight. In the morning, each plate was washed in 1× wash buffer (KPL; Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (MDS Analytical Technologies; Sunnyvale, CA) and then blocked with 1× milk diluent block (MDB; KPL) for 30 min at 37 °C. The plates were then washed again, and 100 µL of 0.125 µg/mL biotinylated HA33A antigen was added to each well. The plates were incubated for 1 h at room temperature. HRP-labeled streptavidin (KPL) was diluted to 0.1 µg/mL in 1× MDB, 100 µL of which was added to each well and incubated at room temperature for 1 h. After plates were washed, 100 µL of room-temperature ABTS 1-component HRP substrate (KPL) was added to each well. After 9 min at room temperature, the optical density at the 405 nm light wavelength was determined using a Synergy H4 hybrid multimode microplate reader (BioTek; Winooski, VT). Data analysis was performed using Prism software, version 5.00 for Windows (GraphPad Software; San Diego, CA).

2.2.7 SPR Methodology

SPR is a method used to determine the kinetic parameters of an antibody–antigen interaction. It is a rapid means for monitoring biomolecular interactions through the excitation of surface plasmons, which results when polarized light is shone through a prism on a sensor chip with a thin metal film coating. The metal film acts as a mirror and reflects the light. When the angle of light shining through the prism is changed, the intensity of the reflected light also changes. These intensity differences can be monitored and recorded. Although the refractive index at the prism side of the chip does not change, the refractive index in the immediate vicinity of the metal surface does change when accumulated mass (bound proteins) adsorbs onto the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this shift of the SPR angle provides information about the kinetics of the protein adsorption on the surface. The SPR software can then be used to accurately analyze the association and dissociation rate constants (k_a and k_d, respectively) for the antibody interactions and to calculate the overall equilibrium dissociation constants (K_D values) between antibodies and antigens.

2.2.7.1 Thermostability Testing

On a Biacore T200 system (GE Healthcare Life Sciences), 6500 response units (RU) of HA33A were tethered to one flow cell of a Biacore CM5 chip using standard amine coupling chemistry. After thermal stress testing was performed, samples were centrifuged (5 °C, 2000×*g*, 5 min). The analyte was run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated antibody at 400, 350, 300, 250, 200, 150, 100, and 50 nM and then plotting the respective maximum analyte-binding capacities of the surface (R_{Max}) in response units. Unheated and heated samples were diluted 1:90 and 1:180 so that the time 0 control points would fall on the linear calibration curve. All samples were run in triplicate. The chip surface was regenerated using an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Data were collected using concentration analysis software (Biacore T200 Evaluation software, GE Healthcare Life Sciences), and the active concentrations of heated samples were recorded. The running buffer used for this experiment was Biacore 1× HBS-EP buffer (GE Healthcare Life Sciences).

2.2.7.2 Kinetic Analysis

Using the Biacore T200 system and 1× HBS-EP running buffer, 102 RU of HA33A was tethered to a Biacore CM5 chip using standard amine coupling chemistry. APE 533.03 was injected across the chip surface for 120 s at a flow rate of 75 µL/min with a 900 s dissociation at 60, 20, 6.67, and 2.2 nM and 700 pM. The chip surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 30 µL/min with a 60 s stabilization period. Data were analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Antibody Concentration Measurements

3.1.1 APE 533.03 Concentration

A_{280} values for APE 533.03 were obtained in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are listed in Table 1.

Table 1. A_{280} Readings for APE 533.03

Replicate No.	A_{280} Value
1	1.765
2	1.779
3	1.757

An average was determined from the A_{280} results, and it was divided by the extinction coefficient of 1.4. The reported concentration was 1.26 mg/mL.

3.1.2 Bot 56 Concentration

A_{280} values for Bot 56 were obtained in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are listed in Table 2.

Table 2. A_{280} Readings for Bot 56

Replicate No.	A_{280} Value
1	7.60
2	7.65
3	7.75

An average was determined from the A_{280} results (the spectrophotometer software automatically divided by the extinction coefficient). The reported concentration was 7.7 mg/mL.

3.2 Molecular Weight and Purity Measurements

3.2.1 APE 533.03 Molecular Weight and Purity

The molecular weight of APE 533.03 was determined (Figure 1) using the Experion Pro260 Analysis Kit. In the figure, the thick band at the top of the second lane corresponds to the IgG antibody heavy chain, and the thinner band at the bottom of the second lane corresponds to the light chain. According to the software, the antibody was 97.1% pure, the heavy chain weighed 61.6 kDa, and the light chain weighed 26.9 kDa.

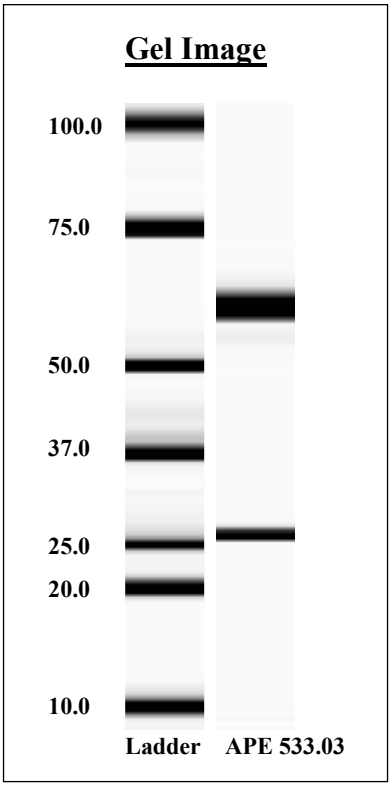


Figure 1. Molecular weight and purity of APE 533.03. Digital gel of APE 533.03 IgG produced by the Experion Pro260 system. The thick band at the top of the right lane corresponds to the APE 533.03 heavy chain, and the thinner band at the bottom of the second lane corresponds to the light chain.

3.2.2 Bot 56 Molecular Weight and Purity

The molecular weight of Bot 56 was measured (Figure 2) using the Experion Pro260 Analysis Kit. In the figure, the thick band at the top of the right lane corresponds to the Bot 56 heavy chain, with a molecular weight of 30.4 kDa. The thinner band at the bottom of the second lane corresponds to the light chain, with a molecular weight of 27.5 kDa. The purity was measured to be 97.3%.

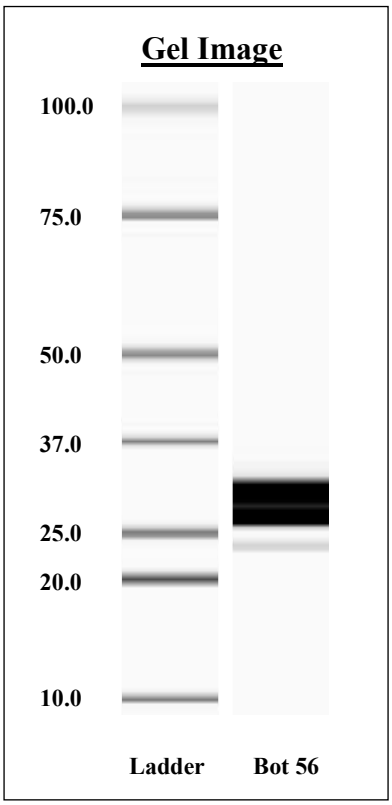


Figure 2. Molecular weight and purity of Bot 56. Digital gel of Bot 56 produced by the Experion Pro260 system. The thick band at the top of the right lane corresponds to the Bot 56 heavy chain, and the thinner band at the bottom of the second lane corresponds to the light chain.

3.3 Protein Behavior by DLS

3.3.1 APE 533.03 Protein Behavior

The APE 533.03 was analyzed (in triplicate) using the DynaPro plate reader. The radius of APE 533.03 was determined to be 5.5 nm, and the polydispersity was 12.4%. Figure 3 shows representative correlation and regularization graphs for the APE 533.03. The correlation graph (panel A) depicts a sigmoidal curve indicative of a valid size distribution. The regularization graph (panel B) illustrates the monodispersity of both samples. Table 3 lists the raw data that were produced for each replicate. Because 99.8% of the mass displayed favorable polydispersity and hydrodynamic radius, the sample preparations were considered to be monodisperse.

Table 3. Features of APE 533.03 in Solution

Replicate No.	Radius (nm)	Polydispersity (%)
1	5.3	11.7
2	5.4	11.8
3	5.6	11.7
4	5.6	14.9
5	5.4	11.9
Average	5.5	12.4

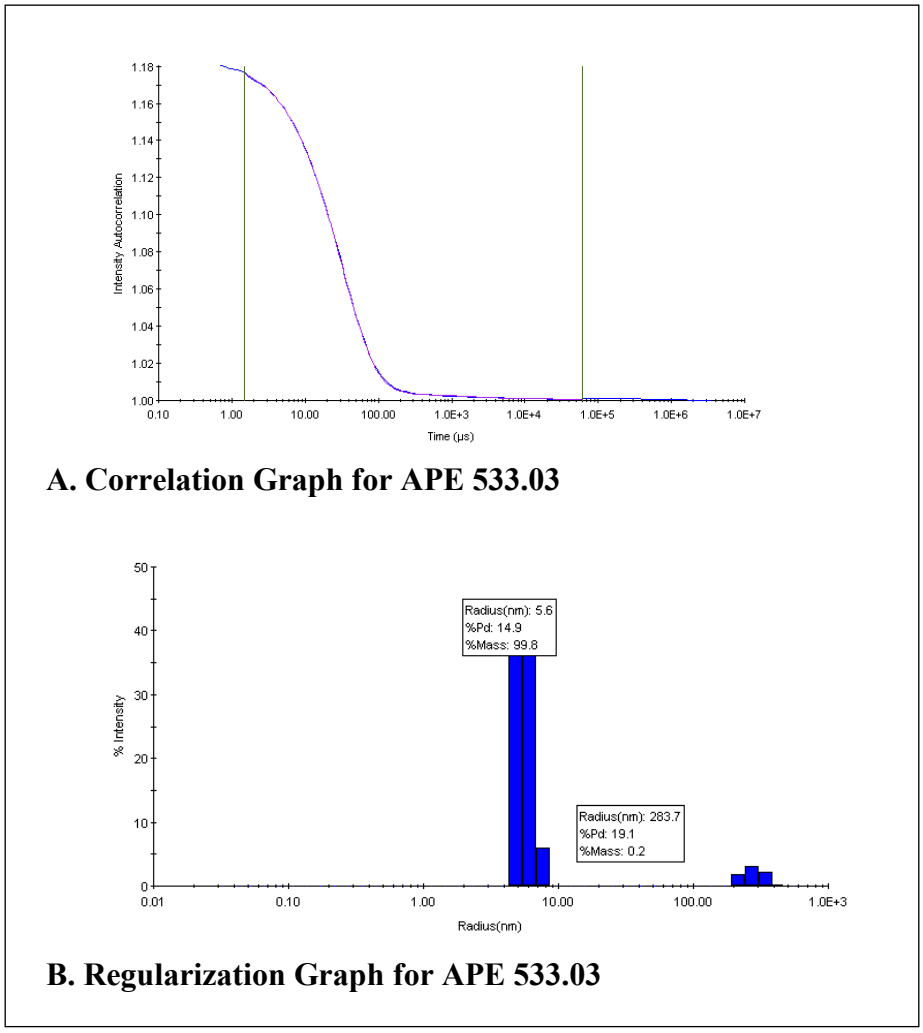


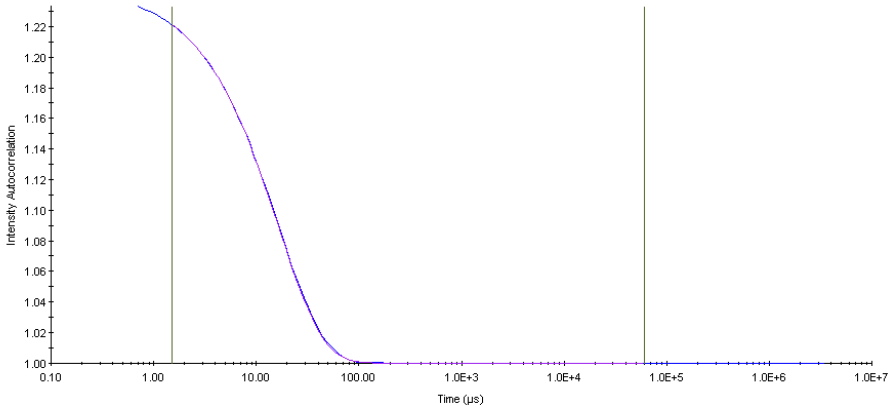
Figure 3. Radius and polydispersity representation of the AnaptysBio thermostable IgG, APE 533.03: (A) correlation graph and (B) regularization graph.

3.3.2 Bot 56 Protein Behavior

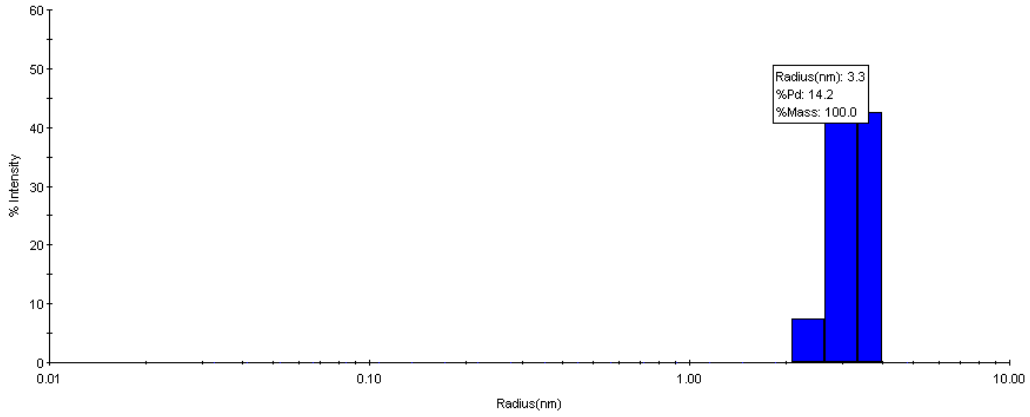
Bot 56 was analyzed using the DynaPro plate reader. The radius was determined to be 3.3 nm, and the polydispersity was 12.0%. Figure 4 contains representative correlation and regularization graphs for Bot 56. The correlation graph (panel A) depicts a sigmoidal curve indicative of a valid size distribution. The regularization graph (panel B) illustrates the monodispersity identified in both samples. Table 4 lists the raw data produced for each replicate. Because 100% of the mass displayed favorable polydispersity and hydrodynamic radius, the sample preparation was considered to be monodisperse.

Table 4. Features of Bot 56 in Solution

Replicate No.	Radius (nm)	Polydispersity (%)
1	3.3	11.7
2	3.3	14.2
3	3.2	11.5
4	3.3	11.6
5	3.2	11.1
Average	3.3	12.0



A. Correlation Graph for Bot 56



B. Regularization Graph for Bot 56

Figure 4. Radius and polydispersity representation for Bot 56:
(A) correlation graph and (B) regularization graph.

3.4 DSC

3.4.1 APE 533.03 Melting Temperature

Readings for APE 533.03 were obtained in duplicate on the MicroCal VP-DSC calorimeter. The peak melting temperatures are shown in Figure 5.

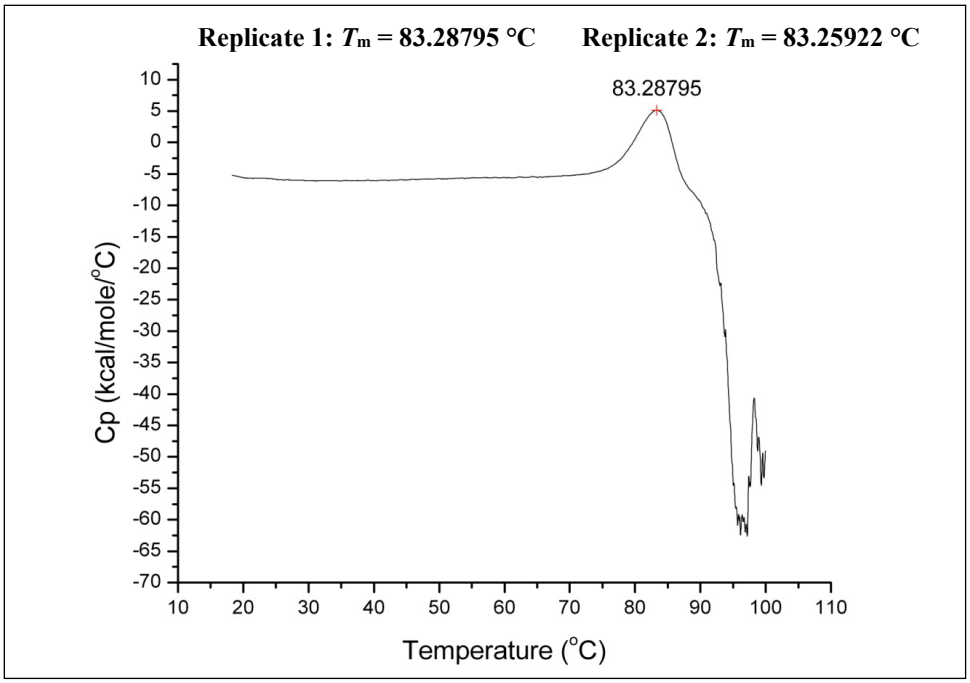


Figure 5. Transition midpoint curve for APE 533.03. T_m was calculated to be 83.3 °C.

3.4.2 Bot 56 Melting Temperature

Readings for Bot 56 were obtained on the MicroCal VP-DSC calorimeter. The peak melting temperature is shown in Figure 6.

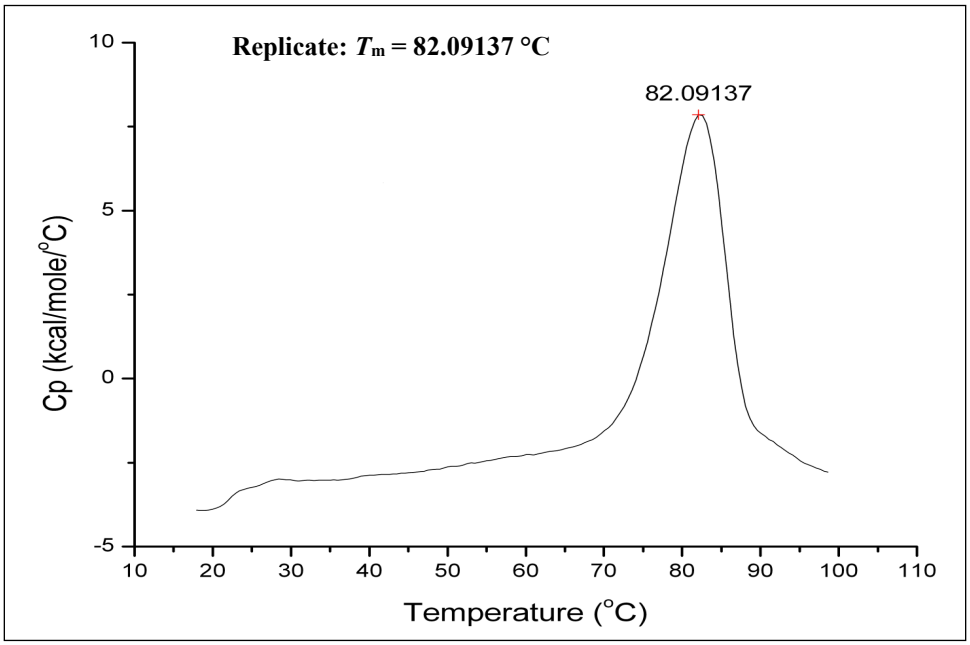


Figure 6. Transition midpoint curve for Bot 56. T_m was calculated to be 82.1 °C.

3.5 Post-Temperature-Stress ELISA

3.5.1 APE 533.03 ELISA

ELISAs were used to test the functional interactions of antibodies and antigens after thermal stress at 75 °C. The ELISA data show that when the APE 533.03 was heated to 75 °C, it maintained all activity across all time periods of thermal stress. The area under the curve for each of the different time points at 75 °C was calculated, averaged, and graphed to depict how the APE 533.03 reacted to thermal stress over time. Figure 7 illustrates that the antibody remained functionally capable of binding to antigen, at about 73% of the original capacity, after a 60 min exposure to 75 °C.

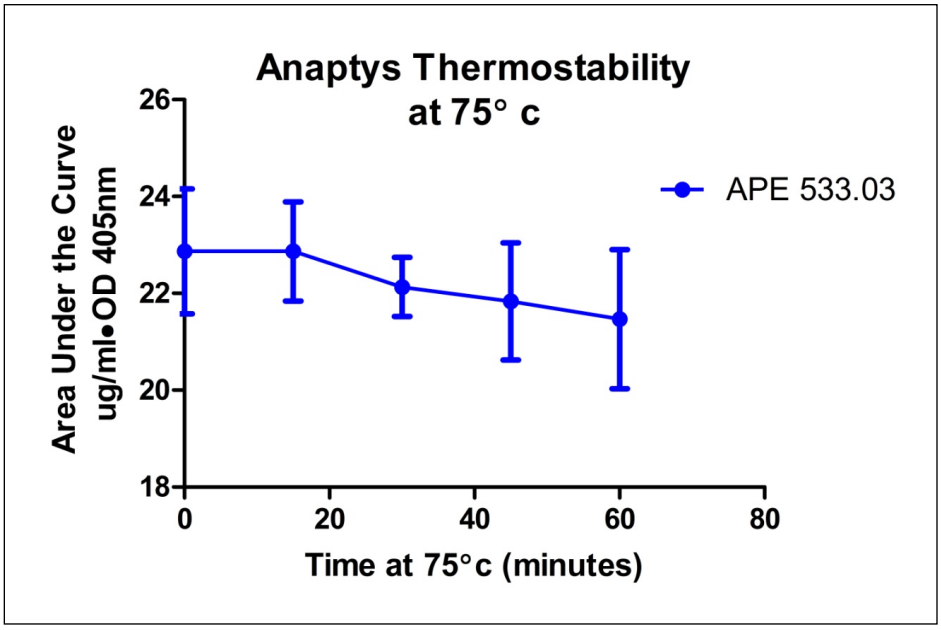


Figure 7. Thermostability of APE 533.03 ELISA. Area under the curve analysis depicts the effect of thermal stress.

3.5.2 Bot 56 ELISA

ELISAs were used to test the functional interactions of Bot 56 as described in Section 3.5.1. The ELISA data (Figure 8) show that when the Bot 56 was heated to 75 °C, it maintained all activity until approximately 30 min of exposure to 75 °C.

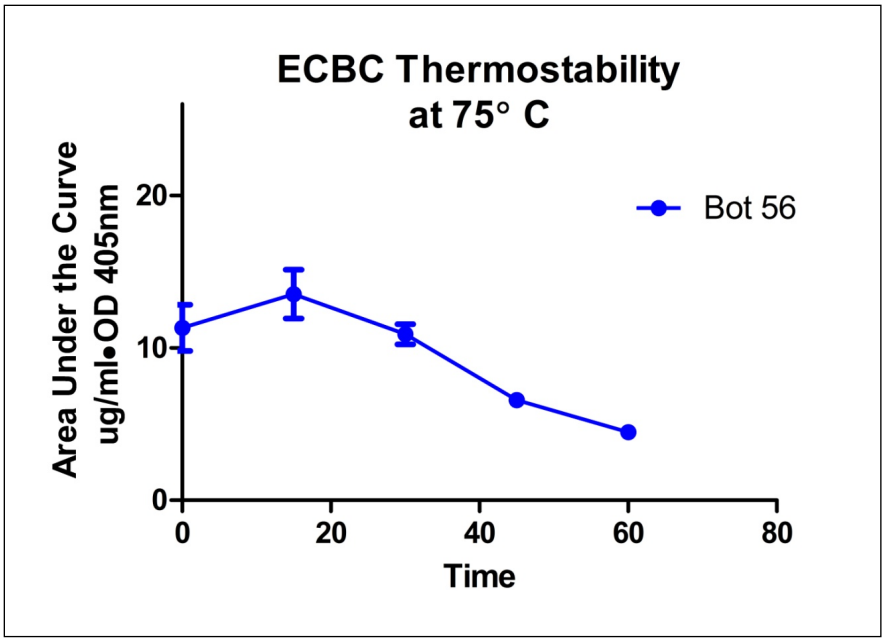


Figure 8. Thermostability of Bot 56 ELISA. Area under the curve analysis depicts the effect of thermal stress.

3.6 SPR

3.6.1 Thermostability Testing by SPR

The functional binding between the antibodies and the antigens was also assessed by SPR after heating to 70 and 75 °C for various time periods. Five tubes of 1 mg/mL APE 533.03 were prepared and heated to 70 or 75 °C for 15, 30, 45, and 60 min time periods and were then quenched on ice. The Biacore T200 system was used to compare the activity of each sample to a calibration curve constructed from unheated sample data (Figures 9 and 10). Data are not shown for Bot 56 at 70 °C.

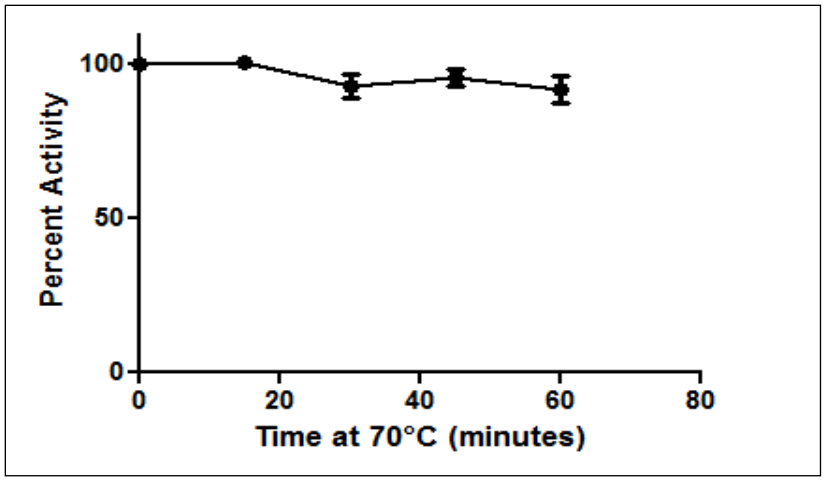


Figure 9. Thermostability of APE 533.03 as assessed using SPR.
APE 533.03 maintained over 90% of its activity after it was heated to 70 °C for 60 min.

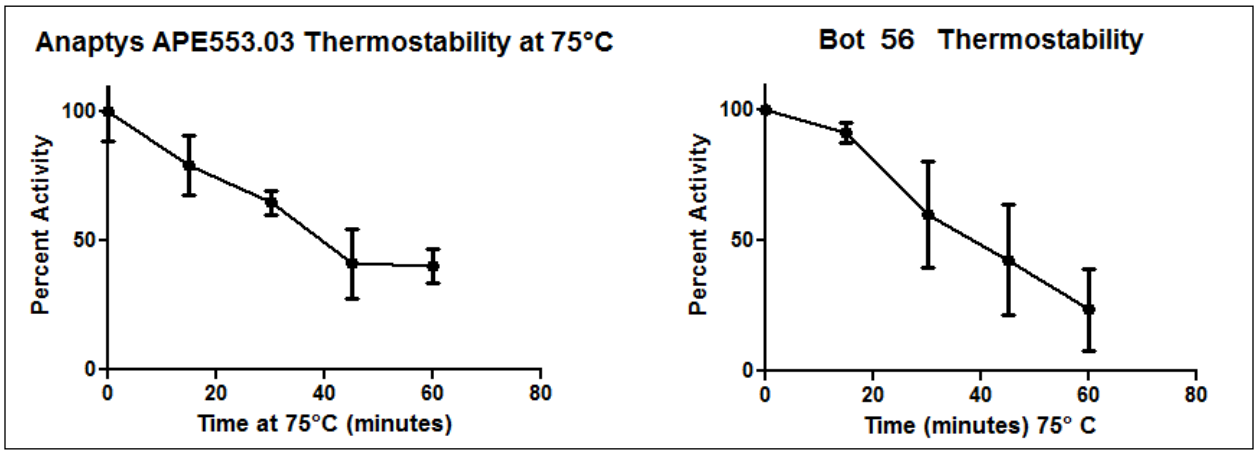


Figure 10. Thermostability of APE 533.03 (left) compared with that of Bot 56 (right) as assessed by SPR. Percent stability was maintained as indicated for the following time periods:
15 min: 72% for APE 533.03 and 83% for Bot 56;
30 min: 64% for APE 533.03 and 53% for Bot 56;
45 min: 43% for APE 533.03 and 42% for Bot 56; and
60 min: 40% for APE 533.03 and 23% for Bot 56.

3.6.2 Kinetic Analysis by SPR

Kinetic analysis of the AnaptysBio APE 533.03 binding to the HA33A antigen was performed as a direct binding SPR experiment on the Biacore T200 system. Results are presented in Figure 11. Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using Biacore T200 software. The K_D of APE 533.03 was determined to be 17.5 pM. Data from similar experiments that were performed using the original Bot 56 are presented in Figure 12. The K_D of Bot 56 was determined to be 4.19 nM; thus, AnaptysBio provided an antibody that was well above the 100-fold improvement threshold.

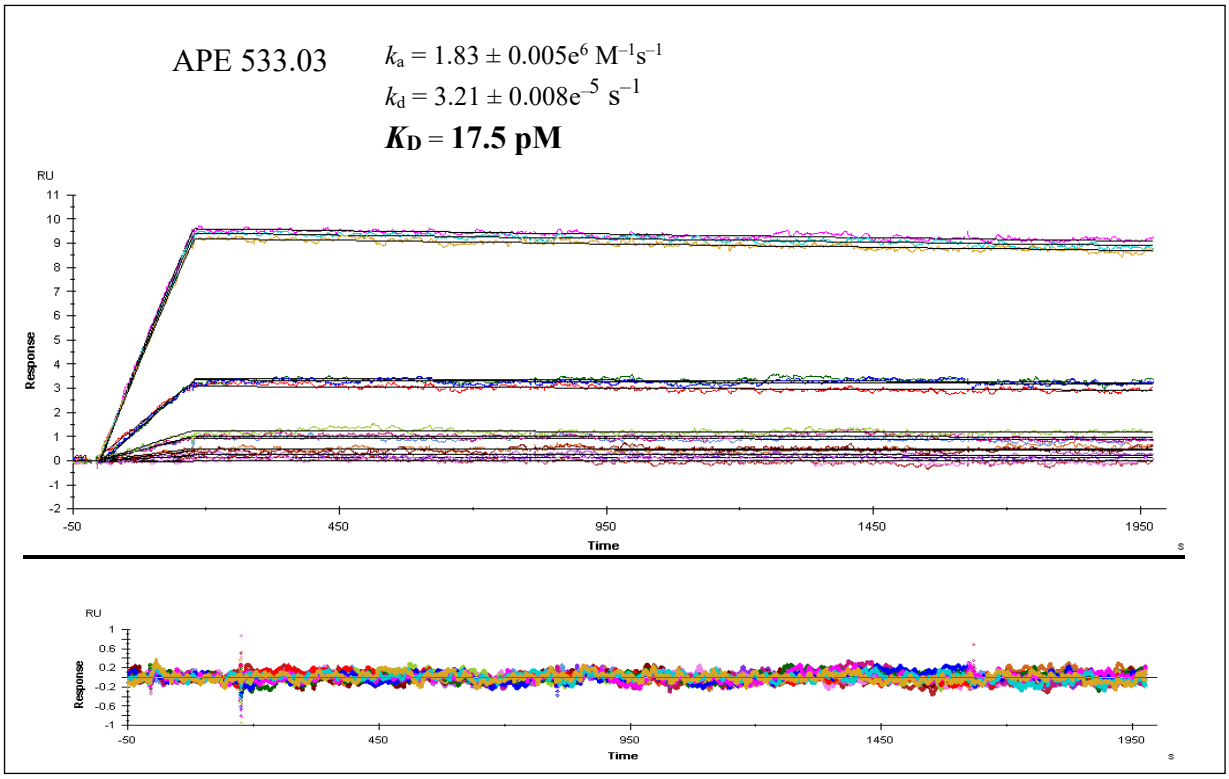


Figure 11. Kinetics of AnaptysBio affinity-enhanced APE 533.03 as determined using a Biacore T200 system.

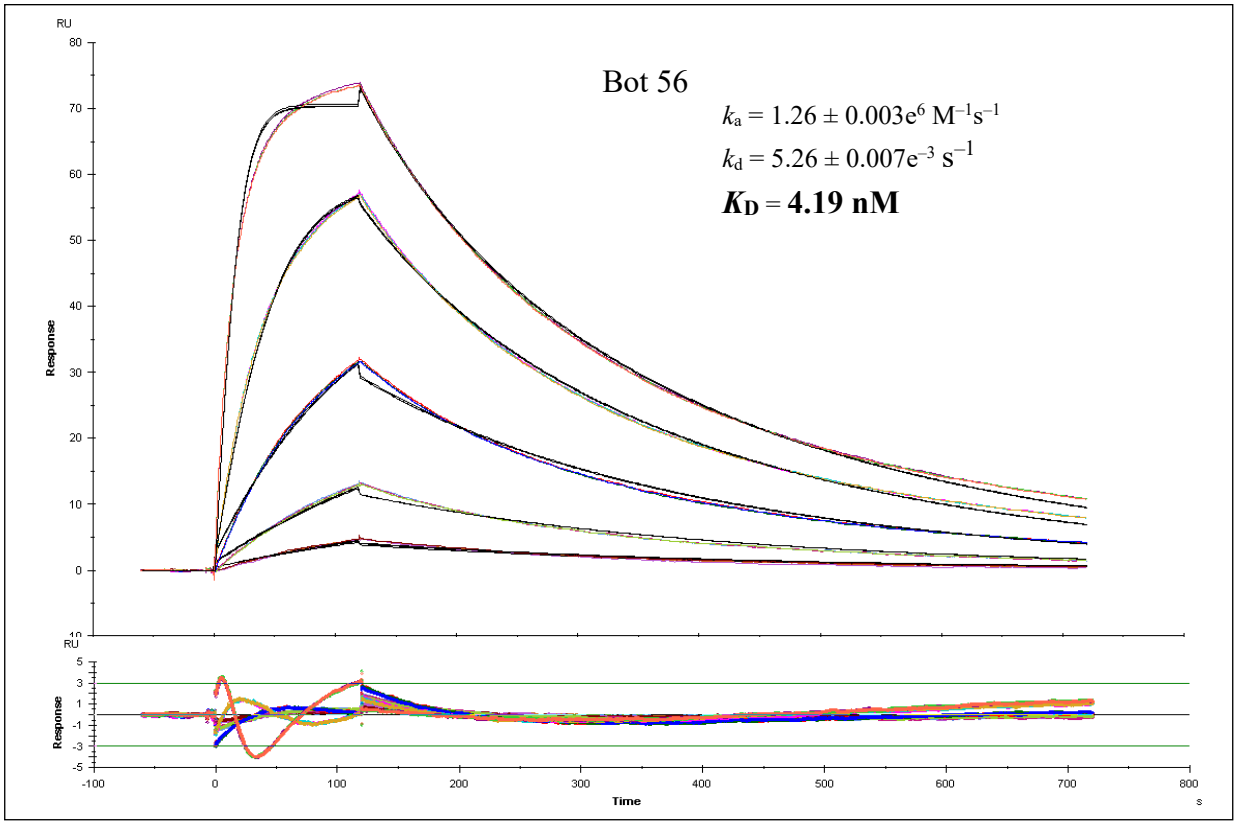


Figure 12. Kinetic fits with residuals of the Bot 56 as determined using a Biacore T200 system.

4. DISCUSSION

In this study, standardized parametric tests were performed that were established during the MS2 scFv antibody DARPA ATP. The performer was able to demonstrate their molecular schemes for improving the thermal stability and affinity of an antibody for its target antigen. Images of the Bot 56 physical characteristics were obtained using the NanoDrop ND-1000, Experion, and DLS measurement platforms. These characteristics were compared with those of the improved antibody submitted by AnaptysBio. Measurements of the APE 533.03 functional characteristics were obtained using the DSC, ELISA, and SPR analytic platforms. These measurements were used to assess the effects of molecular engineering on thermal stability and affinity.

An accurate assessment of protein concentration was critically important for all of the test procedures described in this report. We applied a standard spectrophotometry technique using the NanoDrop ND-1000 system. With this, we obtained the A_{280} values of the samples. A_{280} values are influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, extinction coefficients were used in conjunction with A_{280} readings to determine accurate concentrations.

After concentrations were determined with the NanoDrop ND-1000 spectrophotometer, molecular weight and purity data were collected with the Experion automated electrophoresis system. This system uses microfluidic technology to automate

electrophoresis for protein analysis. The results of Experion analysis of the AnaptysBio APE 533.03 protein fell within the acceptable purity range for use in assay development, and the molecular weight determined by the software was typical for an IgG (Figure 1).

DLS was used in conjunction with the Experion and NanoDrop ND-1000 systems to evaluate how the protein behaved in solution. DLS data indicated the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data established whether the APE 533.03 IgG provided by AnaptysBio was monomeric and monodisperse. Less than 1% of the sample mass appeared to be aggregating in solution (Figure 3). To mitigate the exacerbating effect of freeze–thawing on future sample aggregation and to ensure that all testing would be consistent, the AnaptysBio APE 533.03 samples were aliquoted into single-use vials and centrifuged before use.

SPR was also used to obtain a kinetic analysis of the enhanced AnaptysBio APE 533.03 binding to its target antigen HA33A, to compare binding parameters with those for the original antibody. The k_a and k_d values for APE 533.33 were dramatically improved over those for the originally supplied antibody.

The goal of this study was to modify a single protein that yielded a 100-fold increase in the affinity and a 10 °C increase in the melting temperature of a provided protein. Bot 56, which was provided to each performer, had a 4.19 nM affinity for the recombinant protein HA33A and a melting temperature of 82.1 °C. AnaptysBio provided a 2 g sample of APE 533.03 that produced an affinity of 17.5 pM, which exceeded the 100-fold improvement that was required. This provided protein also had a melting temperature of 83.5 °C. Although this did not improve the temperature by the required amount, this protein did exhibit an excellent improvement in sustained activity at both 70 and 75 °C.

5. CONCLUSION

The DARPA ATP sought to establish methods for rapidly engineering a given antibody reagent to exhibit physical and functional properties that far exceeded those of its native state. This is necessary to expand user confidence in fielding antibody-based detection and diagnostic platforms in environments and operational scenarios that degrade or interfere with the currently available reagents. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP sought to yield antibody reagents that can reliably function in harsh environmental conditions, and thereby increase the sensitivity of a sensor platform to detect lower levels of a threat agent.

This report documents the testing of an improved antibody produced by AnaptysBio. The physical and functional characteristics of IgG APE 533.03 in the ECBC testing pipeline were evaluated. The results were compared with baseline characteristics of the original antibody's physical properties, to include concentration, molecular weight, purity, and state of aggregation in solution as well as functional measures such as binding affinity and thermal stability. The antibody supplied by AnaptysBio exhibited enhanced thermal stability and affinity for binding to the HA33A protein antigen.

REFERENCES

1. Frenzel, A.; Hust, M.; Schirrmann, T. Expression of Recombinant Antibodies. *Front. Immunol.* **2013,** *4,* 217.

2. McConnell, A.D.; Spasojevich, V.; Macomber, J.L.; Krapf, I.P.; Chen, A.; Sheffer, J.C.; Berkebile, A.; Horlick, R.A.; Neben, S.; King, D.J.; Bowers, P.M. An Integrated Approach to Extreme Thermostabilization and Affinity Maturation of an Antibody. *Protein Eng. Des. Sel.* **2013,** *26* (2), 151–164.

3. Buckley, P.E.; Calm, A.; Welsh, H.; Thompson, R.; Kim, M.H.; Kragl, F.J.; Carney, J.; Warner, C.; Zacharko, M. *DARPA Antibody Technology Program Standardized Test Bed for Antibody Characterization: Characterization of an MS2 ScFv Antibody;* ECBC-TR-1356; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Gound, MD, 2016; UNCLASSIFIED Report.

4. *Experion Pro260 Analysis Kit Instruction Manual*; catalog no. 10000975, rev. B; Bio-Rad Laboratories: Philadelphia, PA, 2010.

Blank

ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance of light at 280 nm
ATP	Antibody Technology Program
BGG	bovine γ-globulin
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
HA33A	hemagglutinin 33/A
HRP	horseradish peroxidase
IgG	human immunoglobulin G
k_a	association rate constant
k_d	dissociation rate constant
K_D	equilibrium dissociation constant
kan	kanamycin
LB	Luria broth
mAb	monoclonal antibody
MDB	milk diluent block
RU	response unit
PBS	phosphate-buffered saline
R_{Max}	maximum analyte-binding capacity of the surface
scFv	single-chain variable fragment
sdAb	single-domain antibody
SPR	surface plasmon resonance
T_m	melting temperature

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Combat Capabilities
Development Command Chemical
Biological Center (CCDC CBC)
FCDD-CBR-BC
ATTN: Buckley, P.

CCDC CBC Technical Library
FCDD-CBR-L
ATTN: Foppiano, S.
Stein, J.

Defense Technical Information Center
ATTN: DTIC OA

U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER